LAWRENCE
LIVERMORE
NATIONAL
LABORATORY

LLNL-TR-642206

Isothermal DNA Assay to Detect Drug-Resistant Tuberculosis for Point-of-Care Diagnostics

J. W. Lau, B. R. Baker

August 12, 2013

Disclaimer

This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

Isothermal DNA Assay to Detect Drug-Resistant Tuberculosis for Point-of-Care Diagnostics

JESSICA W. LAU (Clemson University, Clemson SC 29634), BRIAN R. BAKER (Lawrence Livermore National Laboratory, Livermore CA 94550)

August 9, 2013

LLNL-TR-642206

This work was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

ABSTRACT

Tuberculosis (TB) and multidrug-resistant tuberculosis (MDR-TB) remain a global health issue today. There exists an unmet need for a point-of-care TB diagnostic device that is accurate and suitable for use in resource-limited, high-burden settings. This study is the first demonstration of an isothermal DNA assay to identify MDR-TB, with future applications in a low-cost mobile phone diagnostic device. Using loop-mediated isothermal amplification, three primer signatures were identified to have high specificity, high sensitivity, and low limit of detection in amplifying *M. tuberculosis* (MTB) DNA. The use of a dsDNA-intercalating fluorescent dye enabled real-time visualization of the reactions and identification of positive amplifications. Moreover, the assay employs the addition of peptide nucleic acid (PNA) clamps to inhibit the amplification of drug-susceptible MTB DNA and allow the amplification of drug-resistant MTB DNA. Study results showed that combinations of two primer signatures and three PNA clamps inhibit the amplification of drug-susceptible MTB DNA. Results further showed that a relatively high concentration of PNA clamp relative to DNA template is required for inhibition. In future work, PNA clamp design will be improved to increase inhibition of drug-susceptible MTB DNA reactions. Additionally, the effects of the clamps on drug-resistant MTB DNA will be investigated; no amplification is expected.

INTRODUCTION

The bacterium *Mycobacterium tuberculosis* (MTB) causes tuberculosis (TB), the second leading cause of death due to infectious disease worldwide. In 2011, there were 9 million new TB cases and 1.4 million deaths due to TB. Moreover, multidrug-resistant tuberculosis (MDR-TB) constitutes 3.7% of new and 20% of previously treated TB cases globally. MDR-TB is characterized by resistance to rifampicin and isoniazid, two of the most powerful drugs used to treat drug-susceptible TB. Treatment regimens for MDR-TB generally last for 20 months, significantly longer than the 6-month treatment for drug-sensitive TB.[1] There remains an unmet need for a point-of-care TB diagnostic device that is suitable for use in settings with limited resources. We propose a device based on a mobile phone platform, which uses an isothermal nucleic acid amplification assay to identify drug-sensitive and drug-resistant TB.

The World Health Organization (WHO) has identified the continuing need for early TB case detection and effective diagnostic technologies.[2] In 2011, the global case detection rate for TB—the proportion of all cases that were diagnosed—was 66%.[1] The conventional method used for TB diagnosis is sputum smear microscopy, in which patient sputum samples are examined under the microscope for the presence of MTB. This detection method is limited in sensitivity, as only 28% of incident TB cases are determined to be smear positive.[3] Furthermore, the test does not identify between drug-sensitive and drug-resistant strains of bacteria.

Although the Xpert MTB/RIF assay was endorsed by WHO in 2010 and has been described as a landmark development in diagnostic technology,[4] it does not fulfill the requirements for a true point-of-care test.[4,5] The assay uses real-time polymerase chain reaction analysis to detect the presence of MTB in processed sputum samples. Additionally, the reaction identifies rifampicin resistance as a surrogate indicator for MDR-TB.[2] While the assay has high

accuracy, the implementation of this technology is limited by its high cost. Each instrument used to run the assay costs $17,000; each single-use test cartridge costs US$9.98, which can be equivalent to the annual per capita health budget for high-burden countries. [4,5]

The objective of this study is to develop an isothermal DNA assay to detect MTB and drug-resistant MTB, for future application in low-cost point-of-care diagnostics. MTB identification will be achieved with loop-mediated isothermal amplification (LAMP). The technique employs a set of primers that create artificial stem-loops in amplified DNA and a DNA polymerase that exhibits strand-displacement activity.[6] The DNA amplification reaction will be visualized in real-time using a dsDNA-intercalating fluorescent dye.

To distinguish between drug-susceptible and drug-resistant MTB, peptide nucleic acid (PNA) clamps will be added to the LAMP reactions. PNA is a synthetic polymer with an amide backbone and nitrogenous bases, and can be designed to match target DNA sequences. A PNA clamp bound to complementary DNA has a higher melting temperature than that of the corresponding dsDNA duplex; the strong binding of PNA to DNA has been shown to inhibit polymerase displacement activity and DNA amplification. A single base mismatch between the PNA-DNA hybrid causes destabilization, allowing the amplification reaction to proceed.[7] It is expected that PNA clamps designed to bind to wildtype drug-susceptible MTB DNA will inhibition LAMP reactions; because they will not bind as strongly to mutant drug-resistant MTB DNA, amplification will proceed without inhibition.

The specific goals of this study are: 1) to identify primer signatures that amplify MTB DNA with high sensitivity, high speed, and low limit of detection; 2) to characterize the primers' specificity—their ability to distinguish between MTB and genetic near-neighbors (non-tuberculosis mycobacteria, or NTM); and 3) to identify PNA clamps that inhibit amplification of

drug-susceptible MTB DNA and to optimize LAMP reaction chemistry for the addition of PNA clamps.

METHODS AND MATERIALS

Five sets of candidate LAMP primer signatures were designed using LAVA software[8] and purchased from Biosearch Technologies; each set comprised of two inner, two outer, and two loop primers. In each 25µL LAMP reaction, reagents included: 1X isothermal amplification buffer (New England BioLabs, NEB), 8mM $MgSO_4$ (NEB), 0.8M Betaine (Sigma), 1.4mM each dNTP (NEB), 1 x 10^{-3} dilution of PicoGreen (Invitrogen), 8 units *Bst* 2.0 WarmStart DNA polymerase (NEB), 1.6µM inner primers, 0.2µM outer primers, 0.8µM loop primers, and DNA template.

In the initial screening process, each of the 5 primer signatures were tested against 4 different drug-susceptible MTB and 14 NTM DNA extracts. Each DNA extract (BEI, ATCC, and isolates from collaborators at UCSF) was tested in triplicate at 100pg/reaction. Primer signatures that performed well in the initial screening were further tested for limit of detection; the four MTB DNA extracts were tested at concentrations of 100pg, 10pg, 1pg, 100fg, and 10fg per reaction.

Five candidate PNA clamps were designed and purchased from PNA Bio. To optimize LAMP reactions with the addition of PNA clamps, clamp concentrations of 0.8, 1.6, 2.4, and 3.2µM were tested with the candidate primer signatures and four MTB DNA extracts at 100pg and 1pg DNA per reaction.

For negative control reactions (no template control, NTC), water was added in place of DNA extract. A well-characterized set of primers that amplify *E. coli* MG1655 DNA was used as

a positive control. All reactions were run on 96-well plates in a Bio-Rad CFX thermal cycler, for 100 minutes at 63°C.

Data were analyzed with Bio-Rad CFZ Manager software. In a plot of fluorescence versus time, reactions were identified as positive when fluorescence crossed a defined threshold (200 relative fluorescence units, RFU) within the 100 minutes of data collection. The time at which this occurred was determined to be the amplification time of the reaction, or threshold time (T_t).

RESULTS

Table 1 in the Appendix shows the mean amplification times for the initial screening of 5 primer sets against 4 MTB and 14 NTM DNA extracts. Primer sets 1, 2, and 5 showed sensitivity in amplifying MTB DNA. Threshold times for the four MTB DNA extracts ranged from 21 to 32 minutes for primer set 1, 31 to 48 minutes for primer set 2, and 14 to 21 minutes for primer set 5. Out of these three primer sets, primer set 5 MTB reactions were the fastest to amplify and had the highest maximum fluorescence values (around 1000 RFU). In contrast, primer set 3 MTB reactions were slowest to amplify and had the lowest maximum fluorescence values (around 500 RFU). Primer sets 3 and 4 did not amplify all MTB DNA extracts, and were not further tested in limit of detection and PNA clamp studies.

Out of the three primer sets that amplified MTB DNA, primer set 5 also showed good specificity for MTB DNA. As shown in Figure 1 in the Appendix, amplification times for MTB DNA were lower than those for NTM DNA. Primer sets 1 and 2 also showed some degree of specificity in amplifying MTB DNA; most NTM strains had longer amplification times than MTB DNA, but a few NTM reactions had amplification times close to those of MTB DNA.

Primer sets 1, 2, and 5 were tested for limit of detection with the 4 MTB DNA extracts. Amplification times for the MTB 1 DNA extract are shown in Table 2 in the Appendix. For MTB 1, amplification with the three primer sets occurred at all DNA concentrations. For MTB 2, 3, and 4, amplification with the three primer sets occurred at DNA concentrations down to 1000fg per reaction. The limit of detection for each of the three primer signatures was determined to be 1000fg per reaction, indicating that the reactions can identify samples containing as few as 200 copies of the MTB genome. Figure 2 in the Appendix shows the linear trend of amplification times over the range of DNA concentrations; for all three primer sets, amplification times increased as DNA concentration decreased.

Table 3 in the Appendix shows the inhibition of PNA clamps in the amplification of drug-susceptible MTB DNA at 100pg of DNA per reaction, calculated as the difference in amplification times between reactions with 3.2μM PNA and control reactions with no PNA added. Primer set 5 reactions were generally not inhibited by the presence of PNA clamps. PNA clamps 1, 2, and 5 showed inhibition in combination with primer sets 1 and 2. PNA clamps 2 and 5 showed the greatest inhibition of primer sets 1 and 2, and were further tested at 1pg of DNA per reaction; inhibition times are shown in Table 4 in the Appendix. The highest inhibition observed was an average of 18 minutes for all four MTB strains, by primer set 2 in combination with PNA clamp 5. Figure 3 in the Appendix shows the inhibition times of primer set 2 reactions with clamps 2, 4, and 5; increasing inhibition corresponded to increasing GC content in the PNA clamps.

DISCUSSION

Of the three primer sets that showed high sensitivity in amplifying MTB DNA, primer set 5 showed good specificity—a large difference in amplification in times between MTB and NTM DNA was observed. The primer signatures have a low limit of detection, indicating that the LAMP reactions can detect the presence of MTB in samples with a low bacterial load.

Amplification inhibition of drug-susceptible MTB DNA was observed for combinations of two primer sets and three PNA clamps. Future work will involve testing the PNA clamps against drug-resistant MTB DNA; in drug-resistant MTB DNA reactions, no amplification inhibition is expected.

The factors that influence PNA clamp behavior have not been extensively studied. Preliminary results from this study showed a direct relationship between clamp GC content and inhibition time; however, there is a need for further investigation regarding how best to design PNA clamps for applications in LAMP reactions.

The data in this study highlight a potential challenge in the use of PNA clamps in LAMP reactions to distinguish between drug-susceptible and drug-resistant MTB. Inhibition of LAMP reactions for drug-susceptible MTB DNA requires high concentrations of PNA clamp relative to DNA template. There must be a sufficient amount of PNA clamp in the reaction to bind with all of the DNA present; even a small number of DNA molecules without PNA clamps can be amplified and produce a positive reaction. This effect of potential amplification can be limited by using a clamp with high binding affinity; future work will focus on designing and testing clamps that more strongly inhibit drug-susceptible DNA reactions than the ones characterized in this study.

This study demonstrates the feasibility of a rapid, low-cost DNA amplification assay to detect drug-susceptible and drug-resistant TB. The isothermal reaction does not require expensive thermal cycling equipment, and the real-time fluorescence measurements can be optimized for the optics of a mobile phone. Because of these characteristics, the LAMP and PNA assays are suitable for application in a point-of-care diagnostic device.

ACKNOWLEDGEMENTS

This work was supported by the University of California Lab Fees Research Program. This work performed under the auspices of the U. S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. LLNL-TR-642206

REFERENCES

1. World Health Organization. (2012). *Global Tuberculosis Report 2012*. Retrieved from http://www.who.int/tb/publications/global_report/en/
2. Pai, M. & Boyle, D. (2012). *Tuberculosis Diagnostic Technology Landscape: Semi-Annual Update 2012*. Retrieved from http://www.unitaid.eu/en/component/content/article?id=345
3. Boehme, C. C. et al. (2011). Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. *Lancet, 377*, 1495-1505.
4. Lawn, S. D. et al. (2013). Advances in tuberculosis diagnostics: the Xpert MTB/RIF assay and future prospects for a point-of-care test. *Lancet Infectious Diseases, 13*, 349-361.
5. Boyle, D. & Pai, M. (2012). *Tuberculosis Diagnostic Technology Landscape 2012*. Retrieved from http://www.unitaid.eu/en/component/content/article?id=345
6. Parida, M. et al. (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology, 18*, 407-421.
7. Tatsumi, K. et al. (2008). Rapid Screening Assay for *KRAS* Mutations by the Modified Smart Amplification Process. *Journal of Molecular Diagnostics, 10*(6). 520-526.
8. Torres, C. et al. (2011). LAVA: An Open-Source Approach to Designing LAMP (Loop-Mediated Isothermal Amplification) DNA Signatures. *BMC Bioinformatics, 12*(240).

APPENDIX

Strain	Primer set 1		Primer set 2		Primer set 3		Primer set 4		Primer set 5	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
MTB 1	21.8	1.5	31.7	0.7	X	X	42.6	1.2	14.6	0.5
MTB 2	26.5	1.3	39.7	1.5	X	X	54.1	7.8	18.9	2.0
MTB 3	31.3	4.7	47.7	0.4	X	X	X	X	20.5	1.0
MTB 4	28.2	2.1	41.1	0.7	X	X	55.2	5.1	17.5	1.2
NTM 1	93.5	6.2	77.7	15.7	X	X	X	X	X	X
NTM 2	93.2	5.2	72.3	11.7	90.2	—	X	X	X	X
NTM 3	34.3	0.4	63.4	3.8	X	X	X	X	70.5	6.8
NTM 4	61.1	6.1	54.1	0.5	X	X	X	X	67.2	3.3
NTM 5	47.7	3.9	66.3	5.2	X	X	X	X	64.2	6.9
NTM 6	96.6	1.6	85.1	12.2	X	X	95.2	—	97.9	—
NTM 7	47.0	4.5	49.7	5.1	X	X	X	X	87.3	14.7
NTM 8	54.1	5.5	36.1	2.5	X	X	66.6	—	78.4	—
NTM 9	X	X	83.5	12.3	X	X	X	X	X	X
NTM 10	34.9	0.8	59.6	8.3	X	X	X	X	72.4	2.4
NTM 11	39.5	4.6	60.2	10.7	X	X	X	X	81.7	3.0
NTM 12	36.2	2.3	70.7	0.6	X	X	X	X	54.3	4.7
NTM 13	48.3	1.6	59.7	3.7	X	X	X	X	X	X
NTM 14	34.4	3.1	85.0	2.3	X	X	82.0	6.5	39.3	3.4
NTC	63.1	0.7	57.9	—	X	X	89.9	—	55.2	—

Table 1. Mean and standard deviation T_t values (minutes) of five candidate primer signatures. X indicates no amplification in all three reactions. — indicates amplification in only one of three reactions, so no standard deviation was calculated.

DNA concentration per reaction	Primer set 1		Primer set 2		Primer set 3	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
100pg	20.5	0.6	30.2	2.2	13.7	1.5
10pg	23.4	1.7	34.8	1.6	15.5	0.8
1000fg	25.0	2.5	38.9	3.4	17.5	1.2
100fg	31.0	1.3	44.1	2.9	20.1	1.0
10fg	41.2	7.0	49.1	4.2	23.3	3.2
NTC	92.7	4.7	89.5	4.1	X	X

Table 2. Limit of detection results. Mean and standard deviation T_t values (minutes) of three primer signatures for decreasing concentrations of MTB 1 DNA extract. X indicates no amplification in all three reactions.

PNA Clamp	Primer set 1	Primer set 2	Primer set 5
1	3.8	4.4	1.0
2	1.4	6.7	-1.8
3	1.8	0.8	0.9
4	2.7	1.3	3.4
5	6.9	8.3	0.9

Table 3. Average inhibition times (minutes) of four drug-susceptible MTB strains. All combinations of three primer signatures and five PNA clamps tested at 100pg DNA per reaction.

PNA Clamp	Primer set 1	Primer set 2
2	9.3	7.1
5	14.8	18.3

Table 4. Average inhibition times (minutes) of four drug-susceptible MTB strains. All combinations of two primer signatures and two PNA clamps tested at 1pg DNA per reaction.

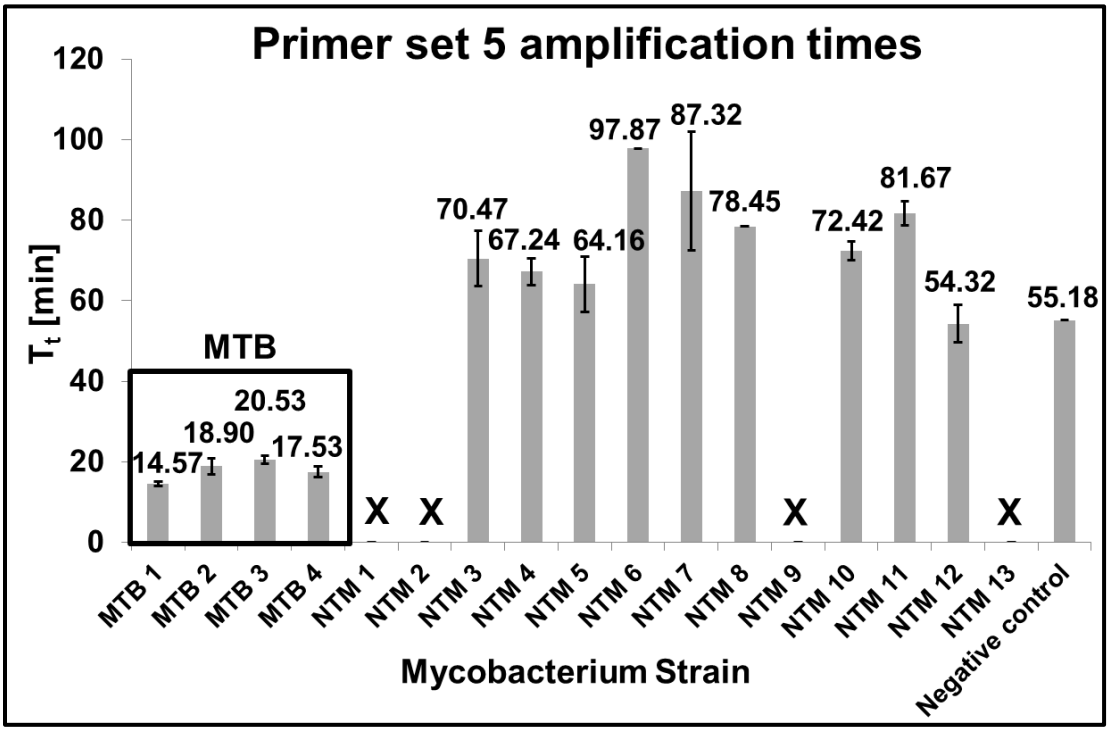

Figure 1. Primer set 5 amplification times for 4 MTB strains (in rectangle), 13 NTM strains, and negative control. X indicates no amplification.

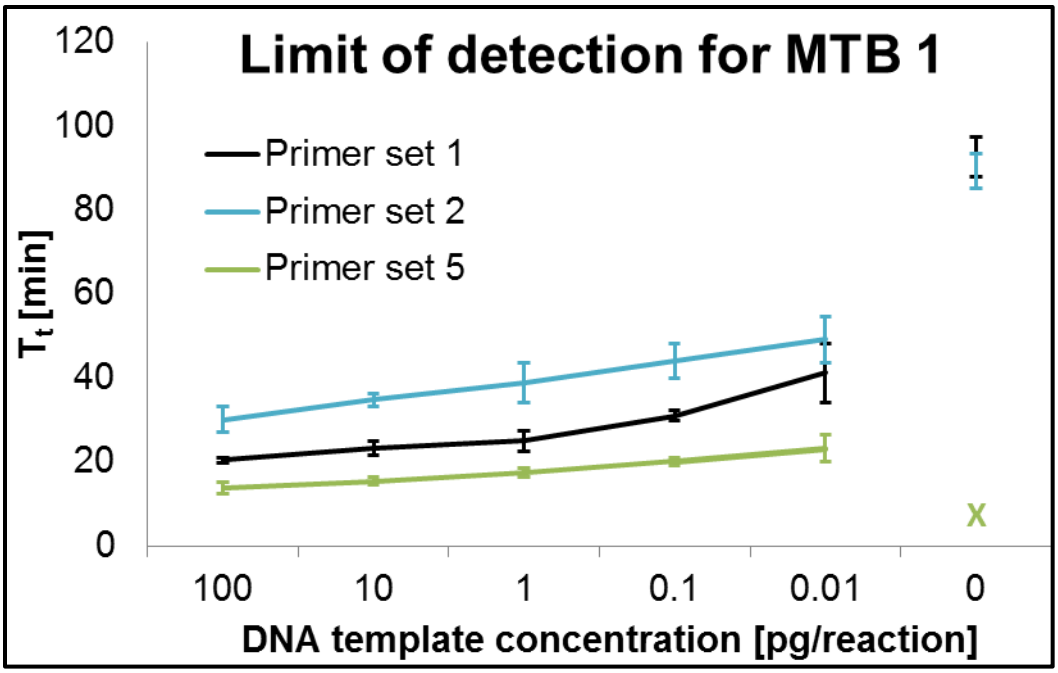

Figure 2. Linear trend of T_t vs. DNA template starting concentration. For all primer sets, amplification times increase as starting DNA concentration decreases. X indicates no amplification.

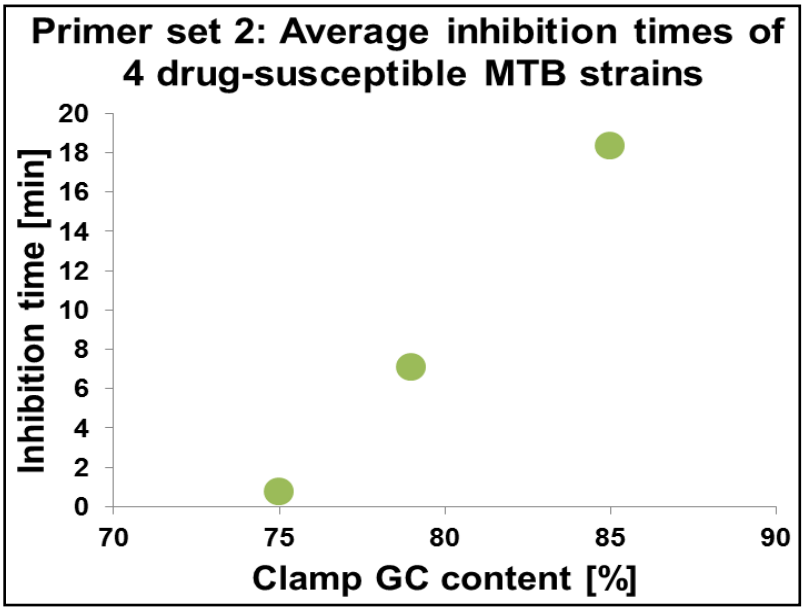

Figure 3. Average inhibition times of primer set 2 increases as clamp GC content increases (clamps 2, 4, 5). 1pg DNA per reaction.